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IMAGING WITH TC-99M LABELED FIBRIN-α-CHAIN PEPTIDE

GOVERNMENT RIGHTS IN THE INVENTION

This invention was made with government support under grant R41 HL 59769-01 (MLT) awarded by the National Institutes of Health. The government has certain rights in the invention.

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims priority under 35 U.S.C. § 119 based upon U.S. Provisional Patent Application No. 60/096,803 filed August 17, 1998.

FIELD OF THE INVENTION

The present invention generally relates to the field of nuclear medicine and, more particularly, to compositions for radiolabeled agents for imaging mammalian tissue or cells, compositions for radiolabeling agents that bind to mammalian tissue or cells, compositions for radiolabeling agents that bind to fibrin, and methods of using said compositions.

BACKGROUND OF THE INVENTION

Development of radioactive agents for "hot spot" imaging of deep venous thrombosis (DVT) and pulmonary embolism (PE) has been the subject of many investigations for more than two decades. (Thakur ML, Coleman RE, Hoist JH, Welch M. Indium-111 labeled platelets: Studies on preparation and *in vivo* and *in vitro* functions. *Thrombosis Research* 9:345-354, 1976; Knight LC.

Radiopharmaceuticals for thrombus detection. *Seminars in Nucl Med* XX:52-67, 1990; Koblik PD, DeNardo GL, Berger HJ. Current status of Immunoscintigraphy in the detection of thrombosis and thromboembolism. *Seminars in Nucl Med* XIX:221-231, 1989; Thakur ML. Potential of radiolabeled antiplatelet antibodies in the detection of vascular thrombi. In: S.C. Srivastava, ed. *Radiolabeled monoclonal antibodies for imaging and therapy*. Plenum Publishing Co., NATO ASI, series 152, 1988; Thakur ML. Scintigraphic imaging of venous thrombosis: A state of the art. *Thrombotic and Hematologic Disorders* 5:29-36, 1992). One approach to "hot spot" imaging has been to radiolabel platelets, which form a major biochemically active constituent of a thrombus. A large number of agents, therefore, have been evaluated that would target platelets on the assumption that radiolabeled platelets will acreed on an occult thrombus and thereby facilitate its detection by external scintigraphy. Platelets have been labeled *in vitro* using such agents as In-111-oxine which internalizes and binds to platelet cytoplasmic components. (Thakur ML et al., 1976). Platelets have also been labeled *in vivo* using radiolabeled proteins or peptides that are specific for platelet surface glycoprotein complex IIb-IIIa (Knight LC, 1990; Koblik PD et al., 1989; Thakur ML, 1988; Thakur ML, 1992; Knight LC, Radcliffe R, Maurer AH, Rodwell JD, Alvarez VL. Thrombus imaging with Tc-99m synthetic peptides based upon the binding domain of a monoclonal antibody to activated platelets. *J Nucl Med* 35:282-288, 1994; Knight LC, Maurer AH, Romano JE. Comparison of Iodine-123-Disintegrins for Imaging Thrombi and Emboli in a Canine Model. *J Nucl Med* 37:476-482, 1996; Pearson DA, Lister-James J, McBride WJ, Wilson DM, Martel LJ, Civitello ER, Dean RT. Thrombus imaging using Tc-99m labeled high potency GPIIb./IIIa receptor antagonist. Chemistry and initial biological studies. *J Med Chem* 39:1372-1382, 1996; Lister-James J, Vallabhajosula S, Moyer BR, Pearson DA, McBride BJ, De Rosch MA. Bush LR, Machac J, Dean RT. Pre-Clinical Evaluation of Technetium-99m platelet receptor-binding platelet. *J Nucl Med* 38:105-111, 1997; Line BR, Crane P, Lazewatsky J, Barrett JA, Cloutier D, Kagan M, Lukasiewicz R, Holmes RA. Phase I trial of DMP444, a new thrombus imaging agent. *J Nucl Med* 37:117P, 1996; Barrett JA, Crocker AC, Damphousse DJ, Heminway SJ, Liu S, Edwards DS, Lazewatsky JL, Kagan M, Mazaika TJ, Carroll TR. Biological evaluation of 99mTc cyclic glycoprotein IIb/IIIa receptor

antagonists in the canine arteriovenous shunt and deep vein thrombosis models: Effects of chelators on biological properties of [99mTc]chelator—peptide conjugates. *Bioconjugate Chem* 7:203-208, 1996). Despite the success achieved with these agents in experimental animals and in limited human subjects, only one agent, AcuTect, the Tc-99m labeled peptide P-280, has recently been approved for clinical use. AcuTect is expected to detect acute but not chronic venous thrombosis (AcuTect. Diatide, Inc. *J Nucl Med* 39(10):19N, 1998) or pulmonary embolism, which may harbor activated platelets only sparingly.

A second approach to "hot spot" imaging has been to radiolabel proteins involved in clot formation. During the vessel wall injury, coagulation proteins are activated sequentially and generate the enzyme thrombin. Thrombin cleaves plasma fibrinogen into fibrin monomers, which then polymerize around the platelets and hold them in place as a clot. Fibrin therefore remains an integral part of DVT, fresh or old, and embolized in the lungs or elsewhere in the body. It is primarily for these reasons that I-125-fibrinogen enjoyed popularity for external detection of DVT for a long time (Knight LC, 1990; Koblik PD et al., 1989; Thakur ML, 1992). However, it is no longer available commercially. Iodine-123-fibrinogen and many antifibrin monoclonal antibodies labeled with various radionuclides have also been evaluated. (Koblik PD et al., 1989). However, for many reasons such as the long circulation times or poor image quality, agents other than I-125-fibrinogen did not make it into common nuclear medicine practice.

A third approach to "hot spot" imaging of DVT and PE is to radiolabel antifibrin peptides. The feasibility of this approach has not been previously investigated. One peptide of particular interest is the N-terminus tripeptide, H-Gly-Pro-Arg-OH, of fibrin-α-chain, which was reported by Laudano and Doolittle to be an inhibitor of fibrinogen/thrombin clotting. (Laudano AP, Doolittle RF. Synthetic peptide derivatives that bind to fibrinogen and prevent the polymerization of fibrin monomers. *Proc Natl Acad Sci* 75:3085-3089, 1978). The investigators observed that H-Gly-Pro-Arg-Pro-OH analog of the tripeptide was an even more potent inhibitor of fibrinogen/thrombin clot by binding to C-terminus portion of the γ-chain of fibrin and preventing fibrin polymerization. More recently, Kawasaki et al prepared several more analogs and found that a pentapeptide, H-Gly-Pro-Arg-Pro-

(Pro-OH) had the highest fibrinogen/thrombin clotting inhibiting activity. (Kawasaki K, Miyano M, Hirase K, Iwamoto M. Amino acids and peptides. XVIII. Synthetic peptides related to N-terminus portion of fibrin α-chain and their inhibitory effect on firbrinogen/thrombin clotting. *Chem Pharm Bull* 41:975-977, 1993).

The present invention comprises composition for diagnostic imaging of mammalian cells and tissue. The composition comprises amino acids joined to a linker, which is bound to a moiety that is chelated to a radionuclide. In one of the embodiments, the present invention is a pentapeptide labeled with Tc-99m, that facilitates imaging of DVT and PE.

DEFINITIONS

"TP 850" means the decapeptide, Gly-Pro-Arg-Pro-Pro-Ana-Gly-Gly-(D)-Ala-Gly (SEQ ID NO:1).

SUMMARY OF THE INVENTION

The present invention comprises a composition for imaging mammalian cells and tissue and method of using said composition.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1. The amino acid sequence and the proposed structure of Tc-99m-TP850.

Fig. 2. A composite of two HPLC elution spectra obtained under identical conditions of solvent composition, flow rate, and column. The x axis in both panels is time in minutes and the y axis is radioactivity peak height in μV. The diagonal line is the percent solvent composition. The upper panel is the elution profile of Tc-99m-TP 850 that was injected into the rabbit, and the lower panel is

that of the urine sample collected from the rabbit 3 hrs later. Note that the major proportion of the radioactivity eluted in the urine has the retention time (Rt) similar to that in the sample injected. The radioactivity at Rt 4 is unbound Tc-99m. The small radioactivity peaks at Rt 6.2 and 9.08 are considered as impurities in the sample. The quantity of the peptide injected was small and was not detectable at 280 nm.

Fig. 3. An anterior image of a rabbit obtained at 3 hr post-injection. A small thrombus induced by stimulating electrode in the right arm (arrowhead) and PE in both upper lobes of the lungs (long arrows) are detectable. Also seen in the right side of the neck (short arrows) is radioactivity accumulated in the incision. The radioactivity in the heart and sinus can be seen.

Fig. 4. An anterior image of a rabbit obtained at 1hr 20 min post-injection. A clot induced by stimulating electrode (clot/blood = 6.5) and the one by thrombine-soaked suture (clot/blood = 3.7) are detectable. In addition, radioactivity in the heart, thyroid, and paranasal sinuses can be seen. Free Tc-99m in preparation was approximately 3%.

Fig. 5. Anterior gamma camera images of a rabbit which was injected with 2 mCi Tc-99m-TP850 2 hr and 30 min previously. A clot due to thrombine-soaked suture in the right (arrow) jugular vein and due to stimulating electrode in the left (arrow) are detectable. The activity due to some free Tc-99m in the thyroid can also be seen. As stated in the text, the electrode clot had 7.1 times more Tc-99m than that in the equal weight of blood and the thrombin clot had 3.6 times more Tc-99m than in the blood. The lower part of the radioactivity is in the heart.

Fig. 6. A composite of three images from one rabbit, in which PE was induced 24 hr prior to the i.v. administration of 2.4 mCi of Tc-99m-TP 850. The scintiphoto in the left panel of the figure was obtained at 1 hr 15 min post-injection in the anterior position. It shows abnormal accumulation of radioactivity in both lungs (arrow). A clot formed spontaneously in the left neck where the incision was made for the placement of the PE introducer sheath is also seen in the scintiphoto given in the left panel of the figure. At the conclusion of in vivo scintigraphy, the heart and lungs were excised, spread for clarity, and then imaged under a gamma camera, as well as x-rayed. The x-ray image (center panel) shows a tantalum

mixed clot in the left lung which corresponds to the shape of a clot seen in the left lung (anterior scintiphoto in the left panel of the figure), as well as to the left lung clot seen in the gamma camera image of the excised lungs and heart given in the right panel of the figure. The clot seen in the right lung, in both in vivo (arrow, left panel) and ex vivo (arrow, right panel) images is not seen by x-ray (center panel) because it is free of tantalum. This indicates that this piece of clot may have formed without tantalum in it and lodged in the right lung. Both lung clots were separated, weighed, and counted for radioactivity. The clot in the left lung had three times more and the one in the right lung had 6.1 times more activity than in the unit weight of blood. This clot in the neck had 3.2 times more activity than in the unit weight of blood. Residual blood radioactivity in the heart (H) can also be seen in the right panel of the figure.

DETAILED DESCRIPTION

Materials and Methods

i) Preparation of peptide

Gly-(D)-Ala-Gly-Gly (SEQ ID NO:4)

For this study, a group of four amino acids, Gly-(D)-Ala-Gly-Gly (GAGG)was chosen as a chelating moiety. Through their NH_2 groups these peptides provide an N_4 configuration for a strong chelation of Tc-99m. Rather than the conventional post-synthesis conjugation, the tetrapeptide chelating moiety permitted the modification of the primary peptide at the C terminus during the synthesis. Furthermore, during the synthesis, an additional amino acid, Aba (4-aminobutyric acid), was inserted as a spacer between the chelating moiety and the primary peptide. The purpose of inserting Aba as a spacer was to minimize any possible steric hindrance resulting from the Tc-99m complex. The synthesis of this modified peptide was one hybrid process which eliminated the multi-step, lengthy, and frequently inefficient conjugation procedure, yet provided a chelating group for a strong chelation of Tc-99m. The resultant decapeptide, Gly-Pro-Arg-Pro-Pro-Aba-Gly-Gly-(D) Ala-Gly which has an expected M.W. of 850, is hereafter referred to as TP 850.

The peptide was custom synthesized (PeptidoGenic Research Co., Inc. Livermore, CA) using a Shimadzu solid phase synthesizer (Shimadzu, Columbia, MD) and separated using HFIsil, C-18, 5 micron preparative HPLC column. Ion spray mass analysis was performed using Perkin Elmer's Sciex APZ I mass spectrometer (Norwalk, CT). Using this chelating moiety and facility several peptides have previously been prepared and labeled with Tc-99m in our laboratory. (Thakur ML, Pallela VR, Consigny PM. Tc-99m-TP 1201 for imaging thromboembolism. *Radiology* 205:267P, 1997; Pallela VR, Consigny PM, Shi R, Thakur ML. Imaging vascular thrombosis with Tc-99m-TP 1300 peptide derived from active domain of thrombospondin. *J Nucl Med* 39:64P, 1998; Pallela VR, Consigny PM, Shi R, Thakur ML. Tc-99m-labeled Fibrin-α-chain peptide analog for imaging vascular thrombosis. *Eur J Nucl Med* 25:878, 1998; Pallela VR, Thakur ML, Consigny PM, Rao PS, Vassileva-Belnikolovska D, Shi R. Imaging thromboembolism with Tc-99m labeled thrombospondin receptor analogs TP-1201 and TP-1300. *Thrombosis Research* 93:191-202, 1999; Pallela VR, Thakur ML, Chakder S, and Rattan S. 99mTc-labeled vasoactive intestinal peptide receptor agonist: Functional studies. *J Nucl Med* 40:352-360, 1999).

ii) *Radiolabeling and quality control*:

Fifty μg of TP 850 was dissolved in 10 μl 10% acetonitrile in water, then 200 μl of 0.1 M Na_3PO_4 were added, followed by 10-30 mCi Tc-99m in 200 μl isotonic saline previously reduced with 100 μg $SnCl_2$ in 10 μl of 0.05 M HCl. Lately, with a new batch of high purity $SnCl_2$ (Sigma Chemicals, St. Louis, MO) we have been able to reduce the $SnCl_2$ to 10 μg. The reaction mixture was then incubated for 30 min in a boiling water bath. The product was examined by HPLC (Rainin, Emeryville,CA) using a reverse phase C-18 column and gradient solvents of 0.1% TFA in water (A) and 0.1% TFA in acetonitrile (B). The gradient was such that at zero minutes solvent A was 90%, and at 30 min solvent B was 100%. The flow rate was 1 ml/min. The HPLC was equipped with a u.v. detector set at 278 nm, a 2" NaI (Tl) gamma counter, and a rate meter.

iii) *Stability of Tc-99m-TP 850*:

The stability of the radiolabeled peptide at 22° C was examined by HPLC for up to 24 hrs as determined by the characteristic retention time of the radioactivity peak. The in vivo stability was examined by injecting approximately 2 mCi Tc-99m-TP 850 preparation, collecting urine 3 hrs later, and analyzing a 20 μl portion of the urine by HPLC.

iv) *Fibrin binding*:

The ability of Tc-99m-TP 850 to bind to rabbit, dog, and human fibrin was examined in vitro. Institutional approval was obtained to draw human blood and to perform all animal experiments. Approximately 10 ml of venous blood was obtained from a healthy human volunteer and from a normal young adult dog and a rabbit. No anticoagulating agent was added to the blood. After the blood was clotted, from each blood sample, one ml serum samples were dispensed in four separate test tubes and approximately 25 μCi of Tc-99m-TP 850 (specific activity approximately 340 Ci/m mol) were added to each tube and the reagents were gently mixed. Thrombin (six i.u.) was then added to the first two test tubes and an equal volume of saline to the other two. The contents were gently mixed and allowed to incubate for 10 min at 37°C. The test tubes were then centrifuged (2000 g x 10 min), the supernatant carefully removed, and the fibrin clots in the first two test tubes were washed twice with 2 ml 0.9% NaCl. Following centrifugation, the washing liquid was combined with the supernatant. Radioactivity associated with the clot and the supernatant were measured and calculated as the percent of total activity found in the compact fibrin clot.

v) *Inhibition of platelet aggregation*:

Seventeen ml of venous blood from a rabbit and a dog were collected in 3 ml Acid Citrate Dextrose A (ACD A), centrifuged at 180 g for 10 min and platelet rich plasma (PRP) was separated. (Thakur ML, Coleman RE, Hoist JH, Welch M. Indium-111 labeled platelets: Studies on preparation and in vivo and in vitro functions. *Thrombosis Research* 9:345-354, 1976). Aggregation studies were performed using a Chronolog (Havertown, PA) aggregometer. For each study, increasing quantities of TP 850 and 4 μM ADP were added to 500 μl PRP containing approximately 1.5 x 10^8 platelets, stirring at 37°C. Aggregation in the absence of TP 850 was considered 100% and IC_{50} values were determined using the quantity of TP 850 that inhibited aggregation by 50%.

vi) *Blood clearance*:

All animal protocols were approved by the Institutional Animal Care and Use Committee and were strictly followed. Blood clearance of the agent was examined in adult New Zealand white rabbits weighing between 3 to 3.5 kg. Each rabbit was anesthetized by an i.m. injection of ketamine (30 mg/kg) and zylaxine (5 mg/kg). Thereafter a 23 gauge catheter was inserted in the right ear artery and connected to a leuer lock (Burron Med. Inc., Bethlehem, PA). The patency of the catheter was maintained by the administration of 6 i.u. heparin per ml of sterile 0.9% NaCl administered through the leuer lock. This catheter was used for drawing 0.5 ml blood samples in duplicate at 1,5,10,15, and 30 min, and then at 1, 2, and 3 hrs after radionuclide injection. Before each sample collection enough blood was withdrawn to replace saline, which avoided the dilution of each blood sample collected.

The marginal vein of the contralateral ear was used for injecting radioactive agents. The radioactivity in the syringe was measured before and after injection to determine the dose injected, and a suitable Tc-99m standard solution was prepared. Blood samples were then weighed, radioactivity counted, and results were

expressed as percent injected dose per gram (% I.D./g) of blood and plotted as a function of time.

vii) *Tissue distribution studies*:

Tissue samples were harvested from three rabbits three hrs after the administration of Tc-99m-TP 850. Tissues were weighed, radioactivity associated with each tissue, and a reference standard solution of Tc-99m prepared at the time of injection was determined. Radioactivity was expressed as % injected dose/g (% I.D./g) of tissue. Results were averaged and standard deviation was determined.

viii) *Inducing DVT*:

Each of the eight adult (male or female) New Zealand white rabbits, weighing between 3-3.5 kg was anesthetized as described above, the right cubital vein and/or jugular vein was exposed, and a stimulating electrode was inserted (Leadley RJ, Humphrey WR, Erickson LA, Shebuski RJ. Inhibition of thrombus formation by Endothelin-1 in canine models of arterial thrombosis. *Thrombosis and Haemostasis* 74:1583-1590, 1995). The electrode was constructed from a 26-gauge stainless steel hypodermic needle bent at a 90° angle and attached to a 30-gauge, Teflon insulated silver coated copper wire. The needle was inserted into the vessel and then gently pulled so that it was in contact with the endothelial lining of the vessel and secured in place with a flared sleeve inserted over the copper wire. The second electrode was applied to the tongue of the rabbit. The stimulating electrode was attached to the anode and the other electrode to the cathode of a power supply. A 450 μA current was then applied and 10 min later 2 mCi of Tc-99m TP 850 (specific activity approximately 510 Ci/m mol) in 2 ml 0.9% solution was injected through a marginal ear vein. Radioactivity in each dose was measured before and after administration and recorded. A suitable reference solution with a known quantity of Tc-99m was also prepared. In two additional rabbits thrombus was induced by inserting a thrombin-soaked suture into

a jugular vein 10 min prior to the administration of Tc-99m-TP 850. Serial gamma camera images of the rabbit, in the supine position, were then obtained for up to four hours, using a GE Starcam gamma camera (GE, Milwaukee, WI) coupled to a low energy parallel hole collimator. For each image a total of 350,000 counts were collected.

ix) *Inducing pulmonary embolism*:

Pulmonary emboli were induced in six additional rabbits. Radio opaque pulmonary emboli were prepared by drawing 0.5 to .75 ml blood, through a 23G butterfly needle inserted in the marginal ear vein, into a one ml syringe containing 15 mg tantalum powder and 6 i.u. of thrombin. The contents of the syringe were then mixed gently and a clot was allowed to form and harden for 20 min. The clot was removed from the syringe and a one cm long piece of the clot was drawn in an introducer sheath (6Fr, Pinnacle, MediTech, Watertown, MA), which was then inserted into a previously isolated jugular vein and advanced into the right atrium. The clot was then flushed from the sheath with isotonic saline. The position of the tantalum containing clots was confirmed by recording a chest x-ray of the animals before the administration of Tc-99m-TP 850 and an x-ray of the excised lungs after sacrifice. Following clot administration and the confirmation of its localization by x-ray, Tc-99m-TP 850 was injected and the rabbits were imaged as described in the previous section. Four animals with PE were allowed to recover from the surgery. Two rabbits were injected with Tc-99m-TP 850 24 hrs later and the other two were injected 48 hours later.

Upon the conclusion of imaging for PE or DVT, each rabbit was given an intravenous injection of heparin (1000 i.u.) and was then euthanized with sodium pentobarbital (100 mg/kg). A blood sample was drawn, and then the lungs and heart were excised, radiographed, and the clots were harvested. The clots and blood were weighed, radioactivity associated with them was counted, and clot/blood ratios were determined.

Results

i) *Peptide radiolabeling, quality control, and stability*:

The purity of the peptide as determined by HPLC analysis was > 90%. The expected M.W. of the peptide was 850, and the one observed by mass spectroscopic analysis was 849.4. The proposed structure of Tc-99m labeled TP 850 is given in **Fig. 1**, which shows that Tc-99m is bound to the chelating moiety with N_4 configuration. The Tc-99m labeling consistently produced > 95% yield. HPLC analysis indicated that > 90% of that activity was eluted in a single peak at retention time (Rt) of seven min. A small quantity (< 5%) of radioactivity was eluted at a Rt of 6.2 min and any unbound Tc-99m at a Rt of 3.5 min. An elution profile is given in **Fig. 2**.

The preparations of Tc-99m-TP 850 were stable at 22°C for 24 hrs. HPLC analysis of a urine sample (**Fig. 2**) collected at three hrs after an injection of Tc-99m-TP 850 showed that the radioactivity elution profile was similar to that of the preparation injected, and that the retention time of the radioactivity peak in the urine sample was similar to that of the radioactivity sample injected. This suggested that the small peptide was not susceptible to a rapid in vivo proteolysis.

ii) *Blood clearance and tissue distribution*:

The blood clearance was biphasic with a $t_{1/2}$-α being approximately four min (20%) and $t_{1/2}$ - β being approximately 13 min (80%). Examination of three hr tissue distribution of Tc-99m-TP 850 indicated that the highest radioactivity was in the kidneys (0.10 ± 0.086 % I.D./g), suggesting that the kidneys were the primary route of excretion. The liver uptake was 0.016 ± 0.014% I.D./g and intestine 0.01 ± 0.009% I.D./g. The blood uptake at this time was only 0.007 ± 0.004% I.D./g. This small proportion of radioactivity in circulating blood facilitated the imaging of vascular thrombi. Radioactivity in all other tissues was unremarkable.

ii) *Fibrin binding and inhibition of platelet aggregation*:

TP 850 radioactivity associated with human, dog, and rabbit fibrin was 42 ± 2 %, 60 ± 39%, and 56 ± 2.5%, respectively. The IC_{50} values for the dog and rabbit platelet aggregation inhibition were 236 μm and 167 μm, respectively. These data justified the use of rabbit as a model for studies with Tc-99m-TP 850.

iii) *Imaging DVT and PE*:

Although Tc-99m-TP 850 cleared rapidly from the blood, cardiac blood pool activity was detectable in all animals at all imaging times. Radioactivity in the sinus was also detectable in all animals studied. This was consistent with the results of Tc-99m-TP1201 and Tc-99m-TP1300, the activated platelet receptor specific thrombospondin analogs studied previously in our laboratory. (Pallela VR, Thakur ML, Consigny PM, Rao PS, Vassileva-Belnikolovska D, Shi R. Imaging thromboembolism with Tc-99m labeled thrombospondin receptor analogs TP-1201 and TP-1300. *Thrombosis Research* 93:191-202, 1999). Unlike these two agents, however, no Tc-99m-TP 850 radioactivity was seen either in the bone or in the bone cartilage. All fresh DVT and PE were detectable by Tc-99m-TP 850 generally within 90-120 min post-injection. Clots that formed spontaneously in surgical incision or ligated vessels were also detectable. Similarly, PE that were formed by a piece of a clot broken off or separated from a clot that was injected into the right atrium were imageable. An example is given in **Fig. 3**, in which an electrode-induced clot in the right forearm, two PE, one in each upper lobe of the lungs, and radioactivity accumulated in the incision was seen. In this animal, the forearm clot to blood radioactivity ratio was 12, and the PE to blood 1.3 (L), and 2.1 (R). The radioactivity associated with the clots was 0.087% I.D./g, 0.006 % I.D./g and 0.007% I.D./g, respectively.

The clot/blood ratios in the rabbits studied ranged from 1.2 to 12. Many times these clots were small and could not be easily separated without the vessel wall or adjoining fatty tissue. Similarly, tantalum was embedded in many PE.

Consequently, the weight contributed by the additional tissue or tantalum, resulted in the low and variable radioactivity per unit weight of clots, PE, or DVT.

Fig. 4 shows an anterior image of a rabbit given Tc-99m-TP 850 one hr and 20 min previously. A clot in the right jugular vein induced by stimulating electrode and the one in the left jugular vein induced by thrombin-soaked suture were detectable. The clot to blood ratios were 6.5 and 3.7, respectively. The clot radioactivity was 0.035% I.D./g and 0.02% I.D./g. In this animal, the radioactivity was also seen in the thyroid due to 3.5% unbound free Tc-99m that was injected.

Fig. 5 shows an anterior image of a rabbit obtained at 150 min after the injection of Tc-99m-TP 850 into which thrombin-soaked suture was placed in the right jugular vein and a stimulating electrode clot was formed in the left jugular vein. Both clots were detectable with the electrode clot to blood ratio of 7.1 and the suture soaked thrombin clot/blood ratio of 3.6. Included in each suture clot was the weight of the suture itself which artificially decreased the clot/blood radioactivity ratios. The radioactivity incorporated into these clots measured 0.046% I.D./g and 0.024% I.D./g of the weight of the clot.

Fig. 6 is an anterior image of a rabbit with PE in both lungs induced 24 hrs previously. The image was positive at one hr and 15 min post-injection of Tc-99m-TP 850. Lungs were excised, imaged, and x-rayed. The location of the clots was corroborated. The clots were then retrieved, weighed, and associated radioactivity was measured. The clot to blood ratios were 6.1 for the right clot and 3.0 for the one in the left clot. The radioactivity in the clot was 0.021% I.D./ and 0.01% I.D./g.

In contrast, 48 hr old clots were neither detectable by scintigraphy nor by x-ray. This suggested that they were lysed and had disappeared. This is consistent with the high fibrinolytic activity in rabbits. (Didisheim P. Animal models useful in the study of thrombosis and antithrombotic agnents. *Prog in Hemostasis and Thrombosis* Spaet TH, ed. Grune and Stratton: New York, pp. 165-197, 1976; Doolittle RF, Omcley JL, and Surgenor DM. Species differences in the interaction of thrombin and fibrinogen. *J Biol Chem* 237:3123, 1962; Gallimore MJ, Nulkar MV, and Shaw JTB. A comparative study of the inhibitors of fibrinolysis in human, dog, and rabbit blood. *Thromb Diath Haemorrh* 14:145-

158, 1965; Hawkey CM, Fibrinolysis in animals. In *The Haemotstatic Mechanism in Man and Other Animals*, MacFarlane RG, ed. Academic Press: London, pp. 143-150, 1979; Mason RG and Read MS. Some species differences in firbrinolysis blood coagulation. *J Biomed Mater Res* 5:121-128, 1971; Craig IH, Bell FP, and Schwartz CJ. Thrombosis and atherosclerosis: The organization of pulmonary thromboemboli in the pig. *Exper Mol Path* 18:290-301, 1973). The influence of any anticoagulant therapeutic intervention has not yet been studied.

Discussion

In the USA, more than 378,000 patients are hospitalized annually for DVT, and more than 103,000 for PE. (Vital and Health Statistics. Series 13: Data from National Health Survey Ditts Publication No. (PHS)95-1783, 1993). These conditions, despite modern techniques, contribute to more than 200,000 deaths every year. The clinical diagnosis of DVT and PE is unreliable (Burke B, Sostman D, Carroll B, and Witty LA. The diagnostic approach to deep venous thrombosis. *Clinics in Chest Medicine* 16:253-1568, 1995;Worsley DF, Alavi A, Palevsky, HI. Role of radionuclide imaging in patients with suspected pulmonary embolism. *Radiologic Clinics of North America* 31:849-859, 1993), and PE is an often underestimated, underdiagnosed, and undertreated disease. (Janata-Schwatzek K, Weiss K, Riezinger I, Bankier A, Domanovits H, Seidler, D. Pulmonary Embolism: Diagnosis and treatment. *Seminars in Thrombosis and Hemostasis* 22:33-52, 1996).

Venography is invasive and other modalities have limitations. Spiral CT, MRI, and ventilation-perfusion (VQ) scans remain the leading diagnostic tools for its diagnosis. In spiral CT, a number of interpretive pitfalls exist in assessing images of PE and MRI is not likely to replace CT. Although CT has better resolution and less sensitivity to moving lung artifacts, its pitfalls, and use of frequently allergic contrast agents have led investigators to rely upon VQ scanning. VQ scanning itself is a cold spot imaging techniques and can only predict low or high probability of PE. For many clinicians this type of diagnosis is inadequate.

In principle, external scintigraphic techniques aided by the use of a suitable radiopharmaceutical can provide hot spot images and can fulfill the need since scintigraphic techniques are non-invasive, and can scan the entire body of a patient without unreasonable inconvenience or added morbidity to the patient.

During the past few years, a large number of radiopharmaceuticals have been investigated as potential agents to localize DVT or PE. Since thrombi are largely composed of fibrin, platelets and other cells entrapped in the fibrin network, much attention was drawn to the use of radioiodine labeled fibrinogen and In-111 labeled platelets.

In many ways, radiolabeled platelets should be a simple and ideal agent, for they form a major and the most biologically active constituent of a thrombus. However, radiolabeled platelets have been less attractive largely due to their long life span (8 days) that elevated background radioactivity for several days after their administration. The slow clearance of radioactivity causes delay in diagnosis due to suboptimal lesion to background radioactivity ratios. The platelets must also be labeled in vitro which requires skilled personnel. Furthermore, in the presence of anticoagulant therapy, heparin in particular, when accretion of fresh platelets is impeded, In-111 platelet scintigraphy is less successful. An array of antibodies, the majority of them specific for IIb and IIIa glycoprotein complex on the platelet surface, have also been investigated. Success with these has been limited for a variety of reasons including the lack of specificity, unfavorable pharmacokinetics or cumbersome preparation of the agent. The pros and cons of these and other agents have been described by Knight, Thakur, and Koblik et al.

Prompted by the advancements in science and technology of molecular biology, recent development of radioactive agents for non-invasive diagnosis of thromboembolism are centered around the use of Tc-99m labeled peptides specific for resting or activated platelets. Peptides are smaller in size and easier to produce than monoclonal antibodies. They are expected to clear more rapidly from circulation than radiolabeled proteins, less likely to induce any immunological reaction, yet in most cases they enjoy as high a receptor specificity and binding constants as the monoclonal antibodies. Because of the physical characteristic of Tc-99m, the Tc-99m labeled peptides have become even more attractive biomolecules for diagnostic imaging than antibodies labeled with In-111.

Technetium-99m is easy to obtain worldwide, inexpensive, and decays with gamma ray energy (140 KeV, 90%) that can be efficiently detected by gamma cameras, planar or tomographic. It has a half-life (6 hr) that is long enough to perform examinations before excessive radioactive decay has occurred, yet not too long to persist in the body long after the examinations have been carried out and to give excessive radiation dose to the subject.

All of the peptides evaluated thus far are specific for platelet glycoprotein receptor complex IIb IIIa. Among them, one peptide, Tc-99m-P280 was recently approved by the FDA under the trade name AcuTect. As per the manufacturer's description, the peptide is expected to detect only acute thrombi but not old clots or PE. A primary reason for this is physiologic, in that fresh platelets, to which AcuTect may bind in vivo, seldom accumulate in chronic clots or PE. A different approach to the problem is therefore necessary that will permit imaging of DVT as well as PE.

The coagulation process described earlier generates fibrin monomers that form a substantial part of a clot. The actual quantity of fibrin content may vary from clot to clot, but generally it is expected to be the same as the fibrinogen of the blood which in most adults is as high as five grams per 100 grams of plasma proteins. Since fibrin exists on both the surface and within clots that are forming or dissolving, the development of Tc-99m labeled peptide, specific for fibrin is appealing. Such agents, in principle, can target fibrin at any stage or state of a clot and reliably image it. For this purpose, one peptide of particular interest is the N-terminus fibrin α-chain peptide, H-Gly-Pro-Arg-OH (SEQ ID NO:1), which was reported by Laudano and Doolittle to be an inhibitor of fibrinogen/ thrombin clotting (13). The α-chain of fibrin begins with the same tripeptide sequence in many animal species as well as in humans. These investigators observed that H-Gly-Pro-Arg-Pro-OH (SEQ ID NO:2) analog of the tripeptide was an even more potent inhibitor of fibrinogen/thrombin clotting than the tripeptide itself since it also bound to C-terminus portion of the γ-chain of fibrin and prevented fibrin polymerization. More recently, Kawasaki, et al, prepared several more analogs and found that a pentapeptide, H-Gly-Pro-Arg-Pro-Pro-OH (SEQ ID NO:3) had the highest fibrinogen/thrombin clot inhibiting activity.

Peptides chosen for scintigraphic imaging are modified before they are labeled with a radionuclide of choice. In order to accomplish efficient radiolabeling, most commonly, the presynthesized peptides are conjugated with a metal chelating agent. This is a multi-step process in which peptide functional groups are first blocked, chelating agents are conjugated, and excess of reagents are eliminated. The functional groups from the resultant product are then deblocked, the product is separated using preparative HPLC, and the required product is identified by mass spectroscopic analysis. Not only is the procedure time-consuming, but it can also be frequently inefficient.

The hybrid peptide technique which we developed to label the peptide with Tc-99m, is simple, efficient, and eliminates the drawbacks stated above. The results of our fibrin clot binding and platelet aggregation inhibition studies support the notion that these modifications did not compromise the biological activity of the peptide. These results are consistent with previous findings using biologically active peptides.

The binding of Tc-99m-TP 850 to rabbit fibrin and its IC_{50} value for inhibition of rabbit platelet aggregation observed in this study were high enough to justify the use of the rabbit as a model for imaging experimental clots and PE. All clots, formed by vessel wall injury, by stimulating electrode or by thrombin-soaked sutures implanted in the jugular vein, were detectable by gamma scintigraphy. In general, the clots were small and the radioactivity incorporated into them varied from 0.01% I.D./g to 0.087% I.D./g. This variability was probably due to the presence of non-radioactive tissues or tantalum that were not separated but contributed to the weight of the clots. However, neither the proportion of radioactivity incorporated into the clots, nor the variation of this proportion is uncommon in such animal experiments. Despite the relatively small proportion of radioactivity, the clots were detectable in approximately 90 min after injection.

In experiments, Tc-99m-TP 850 had considerably higher radioactivity uptake on PE than at least two activated platelet specific Tc-99m labeled peptides we had evaluated previously. With Tc-99m-TP 850, all PE were detectable except those that had lysed spontaneously at 48 hr post-placement. The disappearance of the 48 hr old clots was confirmed by the loss of x-ray opacity of these clots which

had been impregnated with tantalum at the time of preparation. The choice of using the rabbit as a model was based upon our supportive in vitro data described previously. However, the plasminogen concentration in rabbits is greater than twice as high as in humans. The fibrinolytic activity in rabbits, therefore, is much higher and leads to a rapid dissolution of these clots.

In principle, an antifibrin agent should be more successful in imaging aged thrombi and may be less susceptible to interference by anticoagulant therapy because in such circumstances more fibrin may be exposed on the clot surface and blood flow around the clot may be greater.

I CLAIM:

1. A composition having formula I or II:

X_1-P-X_2-Z-M (I)

M-Z-X_2-P-X_1 (II)

wherein:

X_1 is from zero to twenty natural or synthetic amino acids;

P is a peptide comprising Gly Pro Arg (SEQ ID NO:2), or an analog or fragment thereof;

X_2 is from zero to twenty natural or synthetic amino acids;

Z is a linker comprising one or more natural or synthetic amino acids; and

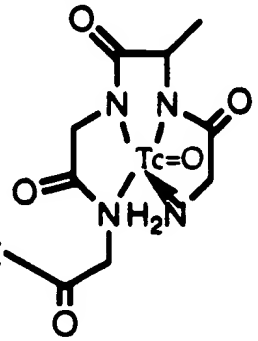
M is a radiolabeling moiety comprised of a chelating moiety capable of complexing with a selected radionuclide.

2. The composition according to Claim 1 comprising SEQ ID NO: 1.

3. The composition according to Claim 1, wherein the radiolabeling moiety is complexed to the radionuclide.

4. The composition according to Claim 3, wherein the radionuclide is technetium-99m.

5. The composition according to Claim 3 having the formula:



Gly-Pro-Arg-Pro-Pro-Aba*-CO-NH

6. The composition according to Claim 1, wherein M comprises Gly –(D)-Ala-Gly-Gly (SEQ ID NO:3) as a chelating moiety for a radionuclide.

7. A method of imaging mammalian cells or tissue, comprising administering a diagnostically effective amount of the composition of Claim 1 to a mammal at a target site and detecting the composition at said target site.

8. The method of Claim 6, wherein said target site is a mammalian thrombus.

9. A method of imaging thrombus in a mammal, comprising:

administering a diagnostically effective amount of a composition that binds to fibrin, said composition having a radiolabeling moiety; and

detecting said composition at a site of said thrombus.

ABTRACT

The present invention involves compositions for radiolabeled agents for imaging mammalian tissue or cells, compositions for radiolabeling agents that bind to mammalian tissue or cells, compositions for radiolabeling agents that bind to fibrin, and methods of using said compositions.